

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE  <b>TRANSMITTAL LETTER TO THE UNITED STATES          DESIGNATED/ELECTED OFFICE (DO/EO/US)          CONCERNING A FILING UNDER 35 USC 371 AND 37 CFR 1.491</b>		ATTORNEY DOCKET NO.  401544 U.S. APPLICATION NO. <b>10/030700</b>
INTERNATIONAL APPLICATION NO. PCT/GB00/02695	INTERNATIONAL FILING DATE July 13, 2000	PRIORITY DATE CLAIMED July 14, 1999
TITLE OF INVENTION CULTURE OF MICROORGANISMS FOR THE SYNTHESIS OF A POLYUNSATURATED FATTY ACID		
APPLICANT(S) FOR DO/EO/US RATLEDGE ET AL.		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 USC 371 and 37 CFR 1.491.		
2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 USC 371 and 37 CFR 1.491.		
3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 USC 371(f)).		
4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).		
5. <input type="checkbox"/> A copy of the International Application as filed (35 USC 371(c)(2)) <ul style="list-style-type: none"> <li>a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</li> <li>b. <input type="checkbox"/> has been communicated by the International Bureau.</li> <li>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</li> </ul>		
6. <input type="checkbox"/> An English language translation of the International Application as filed (35 USC 371(c)(2)).		
7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3)) <ul style="list-style-type: none"> <li>a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</li> <li>b. <input type="checkbox"/> have been communicated by the International Bureau.</li> <li>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li>d. <input type="checkbox"/> have not been made and will not be made.</li> </ul>		
8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 USC 371(c)(3)).		
9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 USC 371(c)(4)).		
10. <input type="checkbox"/> An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 USC 371(c)(5)).		
11. Nucleotide and/or Amino Acid Sequence Submission <ul style="list-style-type: none"> <li>a. <input type="checkbox"/> Computer Readable Form (CRF)</li> <li>b. Specification Sequence Listing on:             <ul style="list-style-type: none"> <li>i. <input type="checkbox"/> CD-ROM or CD-R (2 copies); or</li> <li>ii. <input type="checkbox"/> Paper Copy</li> </ul> </li> <li>c. <input type="checkbox"/> Statement verifying identity of above copies</li> </ul>		
<b>Items 12 to 19 below concern other document(s) or information included:</b>		
12. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. <ul style="list-style-type: none"> <li><input type="checkbox"/> Form PTO-1449</li> <li><input type="checkbox"/> Copies of Listed Documents</li> </ul>		
13. <input checked="" type="checkbox"/> An assignment for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.		
14. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.		
15. <input type="checkbox"/> A substitute specification.		
16. <input type="checkbox"/> A change of power of attorney and/or address letter.		
17. <input checked="" type="checkbox"/> Application Data Sheet Under 37 CFR 1.76		
18. <input checked="" type="checkbox"/> Return Receipt Postcard		
19. <input checked="" type="checkbox"/> Other items or information: Drawings(7 sheets), PCT/RO/101,PCT/ISA210, PCT/ISA224/PCT/IPEA/416,PCT/IPEA/409		

U.S. APPLICATION NO. <b>10/030700</b>		INTERNATIONAL APPLICATION NO. PCT/GB00/02695		ATTORNEY DOCKET NO. 401544	
---------------------------------------	--	---	--	-------------------------------	--

20. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS	PTO USE ONLY
<b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO..... \$1,040.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO..... \$890.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO, but international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... \$740.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... \$710.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1) to (4)..... \$100.00					
<b>ENTER APPROPRIATE BASIC FEE AMOUNT=</b>				\$890.00	
Surcharge of \$130.00 for furnishing the National fee or oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date				\$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	40	-20=	20	x \$ 18.00	\$360.00
Independent Claims	3	- 3 =		x \$ 84.00	\$
<input type="checkbox"/> Multiple Dependent Claim(s) (if applicable)				+\$280.00	\$
<b>TOTAL OF ABOVE CALCULATIONS=</b>				\$1,250.00	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				\$	
<b>SUBTOTAL=</b>				\$1,250.00	
Processing fee of \$130.00 for furnishing English Translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date.				\$	
<b>TOTAL NATIONAL FEE=</b>				\$1,250.00	
Fee for recording the enclosed assignment. The assignment must be accompanied by an appropriate cover sheet. \$40.00 per property				+	\$40.00
<b>TOTAL FEE ENCLOSED=</b>				\$1,290.00	
				Amount to be: refunded	\$
				charged:	\$


a. ☒ A check in the amount of \$1,290.00 to cover the above fee is enclosed.

b. ☐ Please charge Deposit Account No. 12-1216 in the amount of \$ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 12-1216. A duplicate copy of this sheet is enclosed.

**NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.**

SEND ALL CORRESPONDENCE TO:



**23548**

PATENT TRADEMARK OFFICE

*Xavier Pillai*

---

Xavier Pillai, Reg. No. 39,799  
 LEYDIG, VOIT & MAYER, LTD.  
 700 Thirteenth Street, N.W., Suite 300  
 Washington, DC 20005-3960  
 (202) 737-6770 (telephone)  
 (202) 737-6776 (facsimile)

Date: *Jan. 14, '02*

**PATENT**  
Attorney Docket No. 401544/MATHISEN

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Application of:

RATLEDGE et al.

Art Unit: Unassigned

National Phase of PCT/GB00/02695

Examiner: Unassigned

Filed: January 14, 2002

For: CULTURE OF CRYPTHECODINIUM  
COHNII FOR THE SYNTHESIS OF A  
POLYUNSATURATED FATTY ACID

**PRELIMINARY AMENDMENT**

Commissioner for Patents  
Washington, D.C. 20231

Dear Sir:

Prior to the examination of the above-identified patent application, please enter the following amendments and consider the following remarks.

**IN THE TITLE:**

Replace the title with: CULTURE OF MICROORGANISMS FOR THE SYNTHESIS  
OF A POLYUNSATURATED FATTY ACID

**IN THE CLAIMS:**

Please cancel claims 1-33 without prejudice. Add the following claims:

34. (New) A method of culturing a microorganism for the synthesis of docosahexaenoic acid by the microorganism, comprising culturing a microorganism selected from the group consisting of *Crypthecodinium cohnii* and genetically modified variants thereof with a compound selected from the group consisting of carboxylic acids and carboxylate ions, the microorganism using the compound as a carbon source and synthesizing docosahexaenoic acid.

35. (New) The method according to claim 34, wherein said compound is acetic acid or acetate.

36. (New) The method according to claim 34, wherein the compound is the main carbon

source for the microorganism during the culture of the microorganism.

37. (New) The method according to claim 34, wherein the microorganism is cultured in a medium, said use of the compound as a carbon source by the microorganism causing an increase in pH of the medium, and wherein the method further includes, where the compound is a carboxylic acid, addition to the medium of said carboxylic acid, or where the compound is a carboxylate ion, addition to the medium of a carboxylic acid that ionizes to form said carboxylate ion, in response to the increase in pH so as to decrease the pH of the medium.

38. (New) The method according to claim 37, wherein said addition maintains the pH substantially at a predetermined value.

39. (New) The method according to claim 38, wherein the predetermined value is pH 6.5.

40. (New) The method according to claim 37, wherein the pH of the medium is monitored by means that produces a signal that is used to control said addition to the medium.

41. (New) The method according to claim 40, wherein the signal is used to control addition of one or more of a nitrogen source, a phosphorus source, an amino acid, a vitamin, a salt or another growth factor during the culture of the microorganism.

42. (New) The method according to claim 37, wherein said carboxylic acid or said carboxylic acid that ionizes is added to the medium in a mixture comprising a further compound.

43. (New) The method according to claim 42, wherein the further compound is an organic acid.

44. (New) The method according to claim 42, wherein the further compound is a lipid.

45. (New) The method according to claim 42, wherein the mixture is a waste product from an industrial process.

46. (New) The method according to claim 42, wherein the further compound is a nitrogen source, a phosphorus source, an amino acid, a vitamin, a growth factor, a salt or a lipid.

47. (New) The method according to claim 34, wherein prior to said culture with said compound, the microorganism is grown with said compound.

48. (New) The method according to claim 34, wherein the microorganism is cultured with an organic nitrogen source.

49. (New) The method according to claim 48, wherein the organic nitrogen source is yeast extract and the initial concentration of the yeast extract is greater than 7.5 g/l.

50. (New) The method according to claim 49, wherein the initial concentration of yeast extract is 10 g/l.

51. (New) The method according to claim 34, wherein the microorganism is cultured with a salt or an osmoticant.

52. (New) The method according to claim 34, wherein said culture is performed as a batch process or a fed-batch process.

53. (New) The method according to claim 52, wherein the culture is performed for about 4 to about 10 days.

54. (New) The method according to claim 34, wherein said culture is performed as a continuous process or semi-continuous process.

55. (New) The method according to claim 34, wherein the method further comprises extracting oil including docosahexaenoic acid from the microorganism.

56. (New) The method according to claim 34, wherein the method further comprises purification or partial purification of docosahexaenoic acid from the microorganism.

57. (New) The method according to claim 34, wherein the culture does not include a stationary phase.

58. (New) An oil comprising docosahexaenoic acid prepared from a microorganism cultured in accordance with claim 34.

59. (New) An at least partially purified preparation of docosahexaenoic acid prepared from a microorganism cultured in accordance with claim 34.

60. (New) The method according to claim 34, wherein the initial concentration of the compound is about 8 g/l.

61. (New) A method of culturing a microorganism for the synthesis of docosahexaenoic acid by the microorganism, comprising culturing the microorganism with an organic species comprising an acidic group or an ionized form of an acidic group, the microorganism synthesizing docosahexaenoic acid containing carbon from the species.

62. (New) A method of culturing a microorganism for the synthesis of a polyunsaturated fatty acid by the microorganism, comprising culturing *C. cohnii* with an organic species comprising an acidic group or an ionized form of an acidic group, the *C. cohnii* using the species as a carbon source and synthesizing a polyunsaturated fatty acid.

63. (New) An oil comprising the polyunsaturated fatty acid of claim 62.

64. (New) An at least partially purified preparation of the polyunsaturated fatty acid of claim 62.

65. (New) A microorganism cultured in accordance with claim 34.

66. (New) A microorganism cultured in accordance with claim 60.

67. (New) A microorganism cultured in accordance with claim 61.

68. (New) A method comprising using a microorganism according to claim 65 as a food or a food supplement.

69. (New) A method comprising using a microorganism according to claim 66 as a food or a food supplement.

70. (New) A method comprising using a microorganism according to claim 67 as a food or a food supplement.

71. (New) The method according to claim 51, wherein the salt or osmoticant is sea salt.

72. (New) The method according to claim 53, wherein the culture is performed for about 6 to about 9 days.

In re Appln. of RATLEDGE et al.  
Application No. Unassigned

73. (New) The method according to claim 55, which comprises purifying the oil to increase the docosahexaenoic acid content of the oil.

10030700 041402

**REMARKS**

The title has been amended so that it is consistent with the title approved by the International Searching Authority. See copy of attached Form PCT/ISA/224, dated December 14, 2000. Claims 34-73 are currently pending. A set of pending claims is attached.

No new matter has been added by way of this Amendment.

Respectfully submitted,

LEYDIG, VOIT & MAYER, LTD.



Xavier Pillai, Ph.D.  
Registration No. 39,799

Suite 300  
700 Thirteenth Street, N.W.  
Washington, D.C. 20005  
Telephone: (202) 737-6770  
Facsimile: (202) 737-6776  
Date: Jan 14, '02  
XP:jj

10030700 "0440"



PATENT  
Attorney Docket No. 401544/MATHISEN

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

RATLEDGE et al.

Art Unit: Unassigned

National Phase of PCT/GB00/02695

Examiner: Unassigned

Filed: January 14, 2002

For: CULTURE OF  
CRYPTHECODINIUM COHNII FOR  
THE SYNTHESIS OF A  
POLYUNSATURATED FATTY  
ACID

AMENDMENTS TO TITLE MADE VIA PRELIMINARY AMENDMENT

*Amendment to Title:*

~~CULTURE OF CRYPTHECODINIUM COHNII FOR THE SYNTHESIS OF A~~  
~~POLYUNSATURATED FATTY ACID~~ CULTURE OF MICROORGANISMS FOR THE  
SYNTHESIS OF A POLYUNSATURATED FATTY ACID

PATENT  
Attorney Docket No. 401544/MATHISEN

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

RATLEDGE et al.

National Phase of PCT/GB00/02695

Filed: January 14, 2002

Art Unit: Unassigned

Examiner: Unassigned

For: CULTURE OF  
CRYPTHECODINIUM COHNII FOR  
THE SYNTHESIS OF A  
POLYUNSATURATED FATTY  
ACID

PENDING CLAIMS AFTER ENTRY OF PRELIMINARY AMENDMENT

34. A method of culturing a microorganism for the synthesis of docosahexaenoic acid by the microorganism, comprising culturing a microorganism selected from the group consisting of *Crypthecodinium cohnii* and genetically modified variants thereof with a compound selected from the group consisting of carboxylic acids and carboxylate ions, the microorganism using the compound as a carbon source and synthesizing docosahexaenoic acid.

35. The method according to claim 34, wherein said compound is acetic acid or acetate.

36. The method according to claim 34, wherein the compound is the main carbon source for the microorganism during the culture of the microorganism.

37. The method according to claim 34, wherein the microorganism is cultured in a medium, said use of the compound as a carbon source by the microorganism causing an increase in pH of the medium, and wherein the method further includes, where the compound is a carboxylic acid, addition to the medium of said carboxylic acid, or where the compound is a carboxylate ion, addition to the medium of a carboxylic acid that ionizes to form said carboxylate ion, in response to the increase in pH so as to decrease the pH of the medium.

38. The method according to claim 37, wherein said addition maintains the pH substantially at a predetermined value.

39. The method according to claim 38, wherein the predetermined value is pH 6.5.

40. The method according to claim 37, wherein the pH of the medium is monitored by means that produces a signal that is used to control said addition to the medium.
41. The method according to claim 40, wherein the signal is used to control addition of one or more of a nitrogen source, a phosphorus source, an amino acid, a vitamin, a salt or another growth factor during the culture of the microorganism.
42. The method according to claim 37, wherein said carboxylic acid or said carboxylic acid that ionizes is added to the medium in a mixture comprising a further compound.
43. The method according to claim 42, wherein the further compound is an organic acid.
44. The method according to claim 42, wherein the further compound is a lipid.
45. The method according to claim 42, wherein the mixture is a waste product from an industrial process.
46. The method according to claim 42, wherein the further compound is a nitrogen source, a phosphorus source, an amino acid, a vitamin, a growth factor, a salt or a lipid.
47. The method according to claim 34, wherein prior to said culture with said compound, the microorganism is grown with said compound.
48. The method according to claim 34, wherein the microorganism is cultured with an organic nitrogen source.
49. The method according to claim 48, wherein the organic nitrogen source is yeast extract and the initial concentration of the yeast extract is greater than 7.5 g/l.
50. The method according to claim 49, wherein the initial concentration of yeast extract is 10 g/l.
51. The method according to claim 34, wherein the microorganism is cultured with a salt or an osmoticant.
52. The method according to claim 34, wherein said culture is performed as a batch process or a fed-batch process.

53. The method according to claim 52, wherein the culture is performed for about 4 to about 10 days.

54. The method according to claim 34, wherein said culture is performed as a continuous process or semi-continuous process.

55. The method according to claim 34, wherein the method further comprises extracting oil including docosahexaenoic acid from the microorganism.

56. The method according to claim 34, wherein the method further comprises purification or partial purification of docosahexaenoic acid from the microorganism.

57. The method according to claim 34, wherein the culture does not include a stationary phase.

58. An oil comprising docosahexaenoic acid prepared from a microorganism cultured in accordance with claim 34.

59. An at least partially purified preparation of docosahexaenoic acid prepared from a microorganism cultured in accordance with claim 34.

60. The method according to claim 34, wherein the initial concentration of the compound is about 8 g/l.

61. A method of culturing a microorganism for the synthesis of docosahexaenoic acid by the microorganism, comprising culturing the microorganism with an organic species comprising an acidic group or an ionized form of an acidic group, the microorganism synthesizing docosahexaenoic acid containing carbon from the species.

62. A method of culturing a microorganism for the synthesis of a polyunsaturated fatty acid by the microorganism, comprising culturing *C. cohnii* with an organic species comprising an acidic group or an ionized form of an acidic group, the *C. cohnii* using the species as a carbon source and synthesizing a polyunsaturated fatty acid.

63. An oil comprising the polyunsaturated fatty acid of claim 62.

64. An at least partially purified preparation of the polyunsaturated fatty acid of claim 62.

65. A microorganism cultured in accordance with claim 34.

66. A microorganism cultured in accordance with claim 60.

67. A microorganism cultured in accordance with claim 61.

68. A method comprising using a microorganism according to claim 65 as a food or a food supplement.

69. A method comprising using a microorganism according to claim 66 as a food or a food supplement.

70. A method comprising using a microorganism according to claim 67 as a food or a food supplement.

71. The method according to claim 51, wherein the salt or osmoticant is sea salt.

72. The method according to claim 53, wherein the culture is performed for about 6 to about 9 days.

73. The method according to claim 55, which comprises purifying the oil to increase the docosahexaenoic acid content of the oil.

PATENT  
Attorney Docket No. 401544/MATHISEN

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

RATLEDGE et al.

Art Unit: Unassigned

National Phase of PCT/GB00/02695

Examiner: Unassigned

Filed: January 14, 2002

For: CULTURE OF CRYPTHECODINIUM  
COHNII FOR THE SYNTHESIS OF A  
POLYUNSATURATED FATTY ACID

PRELIMINARY AMENDMENT

Commissioner for Patents  
Washington, D.C. 20231

Dear Sir:

Prior to the examination of the above-identified patent application, please enter the following amendments and consider the following remarks.

*IN THE TITLE:*

Replace the title with: CULTURE OF MICROORGANISMS FOR THE SYNTHESIS  
OF A POLYUNSATURATED FATTY ACID

*IN THE CLAIMS:*

Please cancel claims 1-33 without prejudice. Add the following claims:

34. (New) A method of culturing a microorganism for the synthesis of docosaehaenoic acid by the microorganism, comprising culturing a microorganism selected from the group consisting of *Crypthecodinium cohnii* and genetically modified variants thereof with a compound selected from the group consisting of carboxylic acids and carboxylate ions, the microorganism using the compound as a carbon source and synthesizing docosaehaenoic acid.

35. (New) The method according to claim 34, wherein said compound is acetic acid or acetate.

36. (New) The method according to claim 34, wherein the compound is the main carbon

10030700 0140

source for the microorganism during the culture of the microorganism.

37. (New) The method according to claim 34, wherein the microorganism is cultured in a medium, said use of the compound as a carbon source by the microorganism causing an increase in pH of the medium, and wherein the method further includes, where the compound is a carboxylic acid, addition to the medium of said carboxylic acid, or where the compound is a carboxylate ion, addition to the medium of a carboxylic acid that ionizes to form said carboxylate ion, in response to the increase in pH so as to decrease the pH of the medium.

38. (New) The method according to claim 37, wherein said addition maintains the pH substantially at a predetermined value.

39. (New) The method according to claim 38, wherein the predetermined value is pH 6.5.

40. (New) The method according to claim 37, wherein the pH of the medium is monitored by means that produces a signal that is used to control said addition to the medium.

41. (New) The method according to claim 40, wherein the signal is used to control addition of one or more of a nitrogen source, a phosphorus source, an amino acid, a vitamin, a salt or another growth factor during the culture of the microorganism.

42. (New) The method according to claim 37, wherein said carboxylic acid or said carboxylic acid that ionizes is added to the medium in a mixture comprising a further compound.

43. (New) The method according to claim 42, wherein the further compound is an organic acid.

44. (New) The method according to claim 42, wherein the further compound is a lipid.

45. (New) The method according to claim 42, wherein the mixture is a waste product from an industrial process.

46. (New) The method according to claim 42, wherein the further compound is a nitrogen source, a phosphorus source, an amino acid, a vitamin, a growth factor, a salt or a lipid.

47. (New) The method according to claim 34, wherein prior to said culture with said compound, the microorganism is grown with said compound.

48. (New) The method according to claim 34, wherein the microorganism is cultured with an organic nitrogen source.

49. (New) The method according to claim 48, wherein the organic nitrogen source is yeast extract and the initial concentration of the yeast extract is greater than 7.5 g/l.

50. (New) The method according to claim 49, wherein the initial concentration of yeast extract is 10 g/l.

51. (New) The method according to claim 34, wherein the microorganism is cultured with a salt or an osmoticant.

52. (New) The method according to claim 34, wherein said culture is performed as a batch process or a fed-batch process.

53. (New) The method according to claim 52, wherein the culture is performed for about 4 to about 10 days.

54. (New) The method according to claim 34, wherein said culture is performed as a continuous process or semi-continuous process.

55. (New) The method according to claim 34, wherein the method further comprises extracting oil including docosahexaenoic acid from the microorganism.

56. (New) The method according to claim 34, wherein the method further comprises purification or partial purification of docosahexaenoic acid from the microorganism.

57. (New) The method according to claim 34, wherein the culture does not include a stationary phase.

58. (New) An oil comprising docosahexaenoic acid prepared from a microorganism cultured in accordance with claim 34.

59. (New) An at least partially purified preparation of docosahexaenoic acid prepared from a microorganism cultured in accordance with claim 34.



60. (New) The method according to claim 34, wherein the initial concentration of the compound is about 8 g/l.

61. (New) A method of culturing a microorganism for the synthesis of docosahexaenoic acid by the microorganism, comprising culturing the microorganism with an organic species comprising an acidic group or an ionized form of an acidic group, the microorganism synthesising docosahexaenoic acid containing carbon from the species.

62. (New) A method of culturing a microorganism for the synthesis of a polyunsaturated fatty acid by the microorganism, comprising culturing *C. cohnii* with an organic species comprising an acidic group or an ionized form of an acidic group, the *C. cohnii* using the species as a carbon source and synthesizing a polyunsaturated fatty acid.

63. (New) An oil comprising the polyunsaturated fatty acid of claim 62.

64. (New) An at least partially purified preparation of the polyunsaturated fatty acid of claim 62.

65. (New) A microorganism cultured in accordance with claim 34.

66. (New) A microorganism cultured in accordance with claim 60.

67. (New) A microorganism cultured in accordance with claim 61.

68. (New) A method comprising using a microorganism according to claim 65 as a food or a food supplement.

69. (New) A method comprising using a microorganism according to claim 66 as a food or a food supplement.

70. (New) A method comprising using a microorganism according to claim 67 as a food or a food supplement.

71. (New) The method according to claim 51, wherein the salt or osmoticant is sea salt.

72. (New) The method according to claim 53, wherein the culture is performed for about 6 to about 9 days.

In re Appln. of RATLEDGE et al.  
Application No. Unassigned

73. (New) The method according to claim 55, which comprises purifying the oil to increase the docosahexaenoic acid content of the oil.

10030700-011402

REMARKS

The title has been amended so that it is consistent with the title approved by the International Searching Authority. See copy of attached Form PCT/ISA/224, dated December 14, 2000. Claims 34-73 are currently pending. A set of pending claims is attached.

No new matter has been added by way of this Amendment.

Respectfully submitted,

LEYDIG, VOIT & MAYER, LTD.



Xavier Pillai, Ph.D.  
Registration No. 39,799

Suite 300  
700 Thirteenth Street, N.W.  
Washington, D.C. 20005  
Telephone: (202) 737-6770  
Facsimile: (202) 737-6776  
Date: Jan 14, '02  
XP:jj

PATENT  
Attorney Docket No. 401544/MATHISEN

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

RATLEDGE et al.

Art Unit: Unassigned

National Phase of PCT/GB00/02695

Examiner: Unassigned

Filed: January 14, 2002

For: CULTURE OF  
CRYPTHECODINIUM COHNII FOR  
THE SYNTHESIS OF A  
POLYUNSATURATED FATTY  
ACID

AMENDMENTS TO TITLE MADE VIA PRELIMINARY AMENDMENT

*Amendment to Title:*

~~CULTURE OF CRYPTHECODINIUM COHNII FOR THE SYNTHESIS OF A~~  
~~POLYUNSATURATED FATTY ACID~~ CULTURE OF MICROORGANISMS FOR THE  
SYNTHESIS OF A POLYUNSATURATED FATTY ACID

PATENT  
Attorney Docket No. 401544/MATHISEN

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

RATLEDGE et al.

Art Unit: Unassigned

National Phase of PCT/GB00/02695

Examiner: Unassigned

Filed: January 14, 2002

For: CULTURE OF  
CRYPTHECODINIUM COHNII FOR  
THE SYNTHESIS OF A  
POLYUNSATURATED FATTY  
ACID

PENDING CLAIMS AFTER ENTRY OF PRELIMINARY AMENDMENT

34. A method of culturing a microorganism for the synthesis of docosahexaenoic acid by the microorganism, comprising culturing a microorganism selected from the group consisting of *Crypthecodinium cohnii* and genetically modified variants thereof with a compound selected from the group consisting of carboxylic acids and carboxylate ions, the microorganism using the compound as a carbon source and synthesizing docosahexaenoic acid.

35. The method according to claim 34, wherein said compound is acetic acid or acetate.

36. The method according to claim 34, wherein the compound is the main carbon source for the microorganism during the culture of the microorganism.

37. The method according to claim 34, wherein the microorganism is cultured in a medium, said use of the compound as a carbon source by the microorganism causing an increase in pH of the medium, and wherein the method further includes, where the compound is a carboxylic acid, addition to the medium of said carboxylic acid, or where the compound is a carboxylate ion, addition to the medium of a carboxylic acid that ionizes to form said carboxylate ion, in response to the increase in pH so as to decrease the pH of the medium.

38. The method according to claim 37, wherein said addition maintains the pH substantially at a predetermined value.

39. The method according to claim 38, wherein the predetermined value is pH 6.5.

40. The method according to claim 37, wherein the pH of the medium is monitored by means that produces a signal that is used to control said addition to the medium.
41. The method according to claim 40, wherein the signal is used to control addition of one or more of a nitrogen source, a phosphorus source, an amino acid, a vitamin, a salt or another growth factor during the culture of the microorganism.
42. The method according to claim 37, wherein said carboxylic acid or said carboxylic acid that ionizes is added to the medium in a mixture comprising a further compound.
43. The method according to claim 42, wherein the further compound is an organic acid.
44. The method according to claim 42, wherein the further compound is a lipid.
45. The method according to claim 42, wherein the mixture is a waste product from an industrial process.
46. The method according to claim 42, wherein the further compound is a nitrogen source, a phosphorus source, an amino acid, a vitamin, a growth factor, a salt or a lipid.
47. The method according to claim 34, wherein prior to said culture with said compound, the microorganism is grown with said compound.
48. The method according to claim 34, wherein the microorganism is cultured with an organic nitrogen source.
49. The method according to claim 48, wherein the organic nitrogen source is yeast extract and the initial concentration of the yeast extract is greater than 7.5 g/l.
50. The method according to claim 49, wherein the initial concentration of yeast extract is 10 g/l.
51. The method according to claim 34, wherein the microorganism is cultured with a salt or an osmoticant.
52. The method according to claim 34, wherein said culture is performed as a batch process or a fed-batch process.

53. The method according to claim 52, wherein the culture is performed for about 4 to about 10 days.

54. The method according to claim 34, wherein said culture is performed as a continuous process or semi-continuous process.

55. The method according to claim 34, wherein the method further comprises extracting oil including docosahexaenoic acid from the microorganism.

56. The method according to claim 34, wherein the method further comprises purification or partial purification of docosahexaenoic acid from the microorganism.

57. The method according to claim 34, wherein the culture does not include a stationary phase.

58. An oil comprising docosahexaenoic acid prepared from a microorganism cultured in accordance with claim 34.

59. An at least partially purified preparation of docosahexaenoic acid prepared from a microorganism cultured in accordance with claim 34.

60. The method according to claim 34, wherein the initial concentration of the compound is about 8 g/l.

61. A method of culturing a microorganism for the synthesis of docosahexaenoic acid by the microorganism, comprising culturing the microorganism with an organic species comprising an acidic group or an ionized form of an acidic group, the microorganism synthesizing docosahexaenoic acid containing carbon from the species.

62. A method of culturing a microorganism for the synthesis of a polyunsaturated fatty acid by the microorganism, comprising culturing *C. cohnii* with an organic species comprising an acidic group or an ionized form of an acidic group, the *C. cohnii* using the species as a carbon source and synthesizing a polyunsaturated fatty acid.

63. An oil comprising the polyunsaturated fatty acid of claim 62.

64. An at least partially purified preparation of the polyunsaturated fatty acid of claim 62.

65. A microorganism cultured in accordance with claim 34.
66. A microorganism cultured in accordance with claim 60.
67. A microorganism cultured in accordance with claim 61.
68. A method comprising using a microorganism according to claim 65 as a food or a food supplement.
69. A method comprising using a microorganism according to claim 66 as a food or a food supplement.
70. A method comprising using a microorganism according to claim 67 as a food or a food supplement.
71. The method according to claim 51, wherein the salt or osmoticant is sea salt.
72. The method according to claim 53, wherein the culture is performed for about 6 to about 9 days.
73. The method according to claim 55, which comprises purifying the oil to increase the docosahexaenoic acid content of the oil.



CULTURE OF CRYPTHECODINIUM COHNII FOR THE SYNTHESIS OF A POLYUNSATURATED FATTY ACID

The invention relates to a method of culturing a microorganism for the synthesis of a polyunsaturated fatty acid, particularly docosahexaenoic acid, by the microorganism. The invention also relates to oils and preparations of fatty acids, particularly docosahexaenoic acid, prepared from microorganisms cultured in accordance with the method.

Such a method is known from EP 0515460 which describes the culture of the dinoflagellate *Crypthecodinium cohnii* (*C. cohnii*) with glucose. The *C. cohnii* uses the glucose as the carbon source and synthesises docosahexaenoic acid.

According to a first aspect of the invention there is provided a method of culturing a microorganism for the synthesis of docosahexaenoic acid by the microorganism, comprising culturing the microorganism with an organic species comprising an acidic group or an ionised form of an acidic group, the microorganism using the species as a carbon source and synthesising docosahexaenoic acid.

The use of such a species as a carbon source may allow the production of comparable or greater amounts of docosahexaenoic acid, for given culture conditions, compared to culture methods that do not use such a species. Additionally, by choosing suitable, cheap sources of such species, it may be possible to produce docosahexaenoic acid

more cheaply. Further, in the culture method described in EP 0515460, it is necessary to impose a stationary phase in order to achieve satisfactory production of docosahexaenoic acid. Such a phase may not be necessary when such a species is provided as a carbon source.

According to a second aspect of the invention there is provided a method of culturing a microorganism for the synthesis of docosahexaenoic acid by the microorganism, comprising culturing the microorganism with an organic species comprising an acidic group or an ionised form of an acidic group, the microorganism synthesising docosahexaenoic acid containing carbon from the species.

According to the third aspect of the invention there is provided a method of culturing a microorganism for the synthesis of a polyunsaturated fatty acid by the microorganism, comprising culturing *C. cohnii* with an organic species comprising an acidic group or an ionised form of an acidic group, the *C. cohnii* using the species as a carbon source and synthesising a polyunsaturated fatty acid.

The following is a more detailed description of embodiments of the invention, by way of example, reference being made to the appended drawings in which:

Figure 1 is a schematic representation of part of a fermentation apparatus;

Figure 2 is a graph showing certain parameters over time during culture of a first microorganism;

Figure 3 is a similar graph to Figure 2 showing the parameters during culture of a second microorganism;

Figure 4 is a similar graph to Figure 2 showing the parameters during culture of a third microorganism;

Figure 5 is a graph showing dry cell weight over time during culture of the first microorganism after different pretreatments;

Figure 6 is a graph showing lipid produced over time during culture of the first microorganism after the pretreatments of Figure 5;

Figure 7 is a graph showing dry cell weight over time during culture of the first microorganism under different conditions;

Figure 8 is a graph showing lipid production over time during culture of the first microorganism under the different conditions of Figure 7;

Figure 9 is a similar graph to Figure 8 in which lipid production is expressed in a

different manner;

Figure 10 is a graph showing dry cell levels achieved using different initial concentrations of sodium acetate;

Figure 11 is a graph showing lipid levels achieved using different initial concentrations of sodium acetate;

Figure 12 is a graph showing DHA levels achieved using different initial concentrations of sodium acetate; and

Figure 13 includes two graphs showing various values at different time intervals during a culture of *C. cohnii*.

#### Example 1

In Example 1, six microorganisms are cultured, separately, in a growth medium to which an organic acid is added. The microorganisms synthesise docosahexaenoic acid containing carbon from the organic acid.

The culture method will be described in detail for one of the six microorganisms - the strain of *C. cohnii* available from the American Type Culture Collection and identified by the number 30772 (*C. cohnii* ATCC 30772). The remaining five

microorganisms are also strains of *C. cohnii* available from the American Type Culture Collection. They are identified, respectively, by the numbers: 30541; 50298; 40750; 30555; and 30557. These five strains of *C. cohnii* are cultured in an identical manner to *C. cohnii* 30772 and their culture will not be described in detail. Other strains which are also available from the ATCC or from other sources would be expected to behave similarly to those described here.

The culture of *C. cohnii* 30772 is performed in a 5 L fermenter 10 of known type (see Figure 1). The fermenter 10 is provided with a pH electrode 11 that is positioned in the fermenter 10 for monitoring the pH of the growth medium in the fermenter 10. The pH electrode 11 is connected via a control device 12 to a pump 13. The control device 12 is programmed to cause the pump 13 to pump a liquid from a reservoir 14 into the fermenter 10 when the pH electrode 11 detects a pH greater than a predetermined value. This process is described in greater detail below.

The fermenter 10 is also provided with a thermometer (not shown) that is positioned for monitoring the temperature of the growth medium (and also the *C. cohnii* 30772) in the fermenter 10. The thermometer is connected via a control device (not shown) to a heater (not shown). The control device is programmed to control the heater on the basis of the temperature determined by the thermometer so as to maintain the growth medium (and the *C. cohnii* 30772) at a desired temperature within the fermenter 10.

The fermenter 10 is also provided with an aerator (not shown) that is connected to a source of air (not shown) for aerating the growth medium within the fermenter 10. An oxygen sensor (not shown) is positioned for measuring dissolved oxygen concentration in the growth medium within the fermenter 10. A stirrer (not shown) is provided for stirring the growth medium (and the *C. cohnii* 30772). The oxygen sensor is connected to the stirrer by a control device (not shown) for varying the speed of the stirrer in response to the oxygen concentration detected by the oxygen sensor so as to maintain the oxygen concentration at a desired level.

Before initiating the culture, the *C. cohnii* 30772 used to inoculate the fermenter 10 (the "inoculum") is prepared from a standing culture of this organism using a pre-inoculation medium. The pre-inoculation medium contains (initially) glucose (27 g/L), yeast extract (3.8 g/L) and sea salts (25 g/L - commercial preparation purchased from SIGMA, Dorset, UK). Before use, the pH of the pre-inoculation medium is corrected to 6.5 by the addition of 1M KOH and the medium is autoclaved at 121°C for 30 minutes (the glucose is sterilised separately to avoid caramelisation).

The inoculum is prepared by adding 10 ml of the standing culture (4 days old) to a 250 ml flask containing 100 ml of the pre-inoculation medium. The flask is incubated at 27°C while shaking at 200 rpm for 3 days. 70 ml of the incubated mixture is then added to a 2 L flask containing 700 ml of pre-inoculation medium. The 2L flask is then incubated at 27°C while shaking at 200 rpm for 3 days after which the full

content of the 2 L flask forms the inoculum.

The growth medium used for culture in the fermenter 10 contains (initially) sodium acetate (1 g/L), yeast extract (7.5 g/L) and sea salts (25 g/L). Before use, the pH of the growth medium is corrected to 6.5 by the addition of 1M KOH and the medium is autoclaved at 121°C for 1 hour.

Glacial acetic acid mixed with water to give a 50% (v/v) solution is added to the reservoir 14.

The culture is initiated by adding the inoculum into the fermenter 10. The total volume in the fermenter 10 is made up to 3.5 L with growth medium. The fermenter 10 is then closed.

The control device connected to the thermometer and the heater is set so that the heater maintains the growth medium and the *C. cohnii* 30772 at 27°C.

The aerator is set to aerate the growth medium at a rate of 1 volume of air per volume of medium per minute. The control device connected to the oxygen sensor and the stirrer is set so that the dissolved oxygen concentration is maintained above 20% of air saturation. In order to achieve this, the speed of the stirrer varies between 300 to 1000 rpm.

Under these conditions, the *C. cohnii* 30772 grows readily. As the *C. cohnii* 30772 grows it uses the acetate from the growth medium as a carbon source. In fact, it is believed that the *C. cohnii* 30772 takes up acetic acid formed from the acetate - acetic acid being formed in accordance with the equilibrium reaction:  $\text{acetate} + \text{H}^+ \rightleftharpoons \text{acetic acid}$ . Acetate is, of course, a carboxylate ion - it comprises the ionised form of the carboxylic acid group. As the cells use the acetate/acetic acid, the pH of the growth medium increases. As indicated above, the pH of the growth medium is monitored by the pH electrode 11 and when the pH exceeds a pre-set value (slightly greater than 6.5), the control device 12 causes the pump 13 to pump acetic acid solution from the reservoir 14 to the fermenter 10 where it mixes with (and is incorporated into) the growth medium. The addition of the acetic acid solution reduces the pH of the growth medium. The arrangement is such that the pH of the growth medium remains at about 6.5 during the culture.

Acetic acid added in this way dissociates to form, in equilibrium with the acid as indicated above, acetate and protons. The acetic acid derived from the added acetic acid solution, and the acetate formed from this acetic acid, are used as a carbon source by the *C. cohnii* 30772. As indicated above, it is believed that it is acetic acid that is actually taken up by the *C. cohnii* 30772. However acetate may also be taken up (if it is not, it is used as a carbon source by first being converted to acetic acid). The use of the acetic acid/acetate (derived from the added acetic acid) by the *C. cohnii* similarly leads to an increase in the pH of the growth medium and this is counteracted



by the addition of more acetic acid - so as to maintain the pH at about 6.5 - as described above.

By adding acetic acid over a period of time, a considerable amount of acetic acid/acetate can be provided as a carbon source for the *C. cohnii* 30772 while, at any particular moment in time, the concentrations of the acetate and the acetic acid are sufficiently low so as not to be detrimental to growth of the *C. cohnii* 30772. An advantage of this method of cultivation is that it maintains the acetate/acetic acid concentration approximately constant so that it is optimal for the process. The system of adding acetic acid in response to an increase in pH caused by use of acetic acid/acetate is a convenient way of providing acetic acid/acetate as it is needed to replenish acetic acid/acetate used by the microorganisms. The addition of acetic acid in this way allows optimum growth of the *C. cohnii* 30772, a high final biomass (consisting of cells of *C. cohnii* 30772), a high content of oil in the cells and a high proportion of docosahexaenoic acid within the oil.

The acetic acid/acetate is the sole or main carbon source for the *C. cohnii* 30772 - small amounts of other carbon sources may be provided in the yeast extract.

The yeast extract present in the growth medium is provided, primarily, as a source of nitrogen, essential vitamins, amino acids and growth factors. The sea salts are needed for osmo-protection of the *C. cohnii* 30772, which are marine cells in origin.

If, during the culture, the pH falls below 6.5, the pH can be brought back to 6.5 by adding NaOH (2M). This can be done automatically.

During the culture method, samples of the *C. cohnii* 30772 together with growth medium are taken at time intervals. Each sample is analysed to determine dry cell weight, lipid content, docosahexaenoic acid content, and polysaccharide content. The results of these analyses are shown in Figure 2 which shows that dry cell weight, lipid content, docosahexaenoic acid content and polysaccharide content increase with time over the course of the culture method. Significantly, Figure 2 shows that docosahexaenoic acid forms a substantial part of the lipid synthesised by the *C. cohnii* 30772.

As the acetic acid/acetate is a carbon source, the docosahexaenoic acid will contain carbon derived from the acetic acid/acetate.

Growth of *C. cohnii* 30772 proceeds until the cells reach their maximum biomass yield (after about 140 hours of culture in this Example). This is marked by the fact that the pump 13 ceases to operate, indicating that the *C. cohnii* 30772 is no longer consuming acetic acid/acetate. At this stage, no further production of lipid (including docosahexaenoic acid) will occur.

At this stage, the *C. cohnii* 30772 cells and the growth medium are removed from

the fermenter 10 and the biomass (cells of *C. cohnii* 30772) is separated. The oil content of the biomass is extracted using known methods. This oil contains docosahexaenoic acid and is useful as a food or food supplement. The docosahexaenoic acid may be partially or totally purified from the oil and again the purified preparations are useful as food or food supplements. Docosahexaenoic acid in its free acid form, and also esters of docosahexaenoic acid with simple alcohols (e.g. methanol, ethanol, etc) may be prepared from the oil.

Table 1 shows dry cell content, lipid content and the content of docosahexaenoic acid expressed as a percentage of the total fatty acid content of the lipid, as measured after *C. cohnii* 30772 has ceased to grow. Dry cell content and lipid content are expressed as g per L of growth medium and biomass. Table 1 also shows the maximum lipid content in the cells. This is the highest value of lipid content per dry cell weight over the course of the culture. Additionally, Table 1 shows the same parameters measured after culturing the other five strains of *C. cohnii* (strain Nos. 30541, 50298, 40750, 30555, and 30557) under identical conditions.

**Table 1:** Growth of six different strains of *Cryptocodinium cohnii* obtained from the American Type Culture Collection (ATCC) on acetate/acetic acid as described in Example 1.

Strain (ATCC no)	Dry cells (g/L)	Values after 140 h of growth		
		Lipid (g/L)	DHA in total fatty acid from lipid (%)	Maximum lipid in cell (g lipid/ 100g cells)
30772	17	7.48	59	44
30541	45.5	8.32	33	24

50298	45.5	12.3	31	30
40750	17	3.31	46	22
30555	20.7	2.8	30	15
30557	25.6	4.7	40	18

DHA - Docosahexaenoic acid

Table 2 shows further measured parameters and derived values relating to the end points (140 hours of growth) of cultures of the six strains of *C. cohnii*. Specifically, Table 2 shows the amounts of acetic acid utilised and the final volumes of the cultures. Table 2 also shows the yields of biomass, docosahexaenoic acid and lipid.

**Table 2:** Yields of biomass, lipid and DHA of six different strains of *C. cohnii* obtained from the American Type Culture Collection (ATCC) on acetate/acetic acid as described in Example 1.

Strain ATCC no	Acetic acid utilised/ Final volume	Yields of	Yields of	Yields of
		Biomass	DHA	Lipid
		(g/g acetic acid utilised)		
30772	607 g/ 4.2 L	0.118	0.03	0.05
30541	1039 g/ 4.6 L	0.2	0.012	0.037
50298	1107 g/ 5.2 L	0.21	0.018	0.06
40750	340 g/ 4.5 L	0.22	0.026	0.044
30555	498 g/ 4.2 L	0.18	0.007	0.024
30557	754 g/ 4.7 L	0.16	0.01	0.029

As will be seen from Tables 1 and 2, there is a considerable variation in the production and yields of dry cells/biomass, lipid and docosahexaenoic acid between the different

strains of *C. cohnii*. Each of these strains, however, produces potentially useful amounts of docosahexaenoic acid.

Figures 3 and 4 show the same parameters as those shown in Figure 2 - during the culture of *C. cohnii* 30541 and 50298, respectively. The results are similar to those with *C. cohnii* 30772.

It will be appreciated that the culture method of Example 1 constitutes a convenient method for the production of docosahexaenoic acid containing biomass. Moreover, this method may produce comparable or greater amounts of docosahexaenoic acid compared to methods in which glucose is used as the sole carbon source.

It will be appreciated that the method of Example 1 may be varied.

For example, additional nutrients could be added to the acetic acid solution in reservoir 14 so that the nutrients are added to the fermenter 10 together with the acetic acid. Suitable additional nutrients comprise, for example, sources of nitrogen, sources of phosphorus, vitamins and additional salts. Alternatively, instead of adding the nutrients directly to the acetic acid solution in the reservoir 14, the nutrients could be provided in the form of a separate solution in a separate reservoir. Nutrients from the separate reservoir would be pumped into the fermenter 10 by a separate pump. The separate pump would be controlled by the control device 12 and may, for example,

operate at the same time as the pump 13 operates. If additional nutrients are added using a separate pump, the separate pump may be operated to add the nutrients during part, but not all, of the culture period.

Additional carbon source compounds could be added together with the acetic acid. Suitable compounds comprise, for example, oils and lipids containing various precursors of docosahexaenoic acid. Glycerol and glycerophosphate may also contribute to good lipid production.

The acetic acid used could be industrial grade or may be produced by other chemical processes either as a primary or a secondary product. The presence of other aliphatic acids within the acetic acid solution would not be deleterious and may provide additional carbon sources. Waste solutions from industrial processes comprising acetic acid together with other compounds may be suitable for use in place of the acetic acid solution in reservoir 14.

Instead of acetic acid/acetate, other organic species comprising an acidic group or an ionised form of an acidic group, or mixtures of such species, can be used as a carbon source. Preferably, the carbon source is a carboxylic acid or a carboxylate ion, or mixtures of these species. If the carbon source is provided by adding an organic acid to the growth medium, then, preferably the utilisation of the organic acid (and/or its ionised form) by the microorganism results in an increase in the pH of the growth

medium.

The volume of the inoculum may vary. Preferably, this volume will be between 1% and 20% of the initial volume of the culture mixture. More preferably, the volume will be between 5 and 10% of the initial volume of the culture mixture.

Whereas the method of Example 1 is a fed-batch process, it will be appreciated that the culture method may be adapted to be performed as a continuous or semi-continuous process. In this case, the acetic acid and other compounds needed for cell growth, cell maintenance and for oil production would be added into the fermenter throughout the course of the culture. Cells and spent medium would be removed from the fermenter either continuously during the course of the culture or from time to time depending on the volume of the culture or density of the cells. Unlike the culture method described in EP 0515460, which uses glucose as an apparently sole carbon source, the use of acetic acid/acetate as a carbon source does not require the imposition of a stationary phase in order to cause satisfactory production of docosahexaenoic acid. This is particularly advantageous as it will facilitate adaptation of the method to be a continuous or semi-continuous process.

Although the method of Example 1 is performed in a 5 L fermenter 10, it will be appreciated that the method can be performed in larger, industrial scale fermenters. When culture is performed in larger fermenters, it may be advantageous to add the

acetic acid at a plurality of different points into the fermenter. This would aid dispersion of the acid and avoid build-up of any local high concentration of acid.

Whereas, in the method of Example 1, the oxygen concentration is controlled by controlling the stirring speed, it will be appreciated that oxygen concentration may be controlled by controlling the aeration rate or by controlling both aeration rate and stirring speed.

The configuration of the fermenter 10 may be changed and an air-lift fermenter, or a fermenter having appropriate aeration and mixing without mechanical stirring, may be used.

Microorganisms other than *C. cohnii* may be used. When *C. cohnii* is used, it may be used to synthesise other polyunsaturated fatty acids instead of, or in addition to docosahexaenoic acid.

The initial concentration of sodium acetate in the growth medium may be varied. It may be between 0.5 g/L and 20 g/L. Preferably, it will be between 1 g/L and 10 g/L.

The pH of the growth medium may be maintained at values other than 6.5 during the culture. The pH may be maintained at values between 5 and 8. Preferably, the pH is maintained between 6 and 7.5. The acceptable and preferred pH values will depend



on the microorganism used.

### Example 2

In the method of Example 2, *C. cohnii* 30772 is grown, prior to culture in the fermenter 10 as described above in Example 1, with acetic acid/acetate. It is hypothesised that this will allow the cells to adapt to acetic acid/acetate so that they will grow more quickly during culture in the fermenter 10.

The method of Example 2 is similar to the method of Example 1 with the exception of the preparation of the inoculum. The inoculum is prepared by adding 10 ml of the standing culture of *C. cohnii* 30772 to a 250 ml flask containing 100 ml of the pre-inoculation medium described above in Example 1. The flask is incubated at 27°C while shaking at 200 rpm for 3 days. After 3 days, 70 ml of the incubated mixture is added to a 1 L fermenter and the volume in the 1 L fermenter is made up to 700 ml with the growth medium described above in Example 1. The 1 L fermenter is then closed and the fermenter is heated and aerated in the same manner as the 5 L fermenter 10 of Example 1. Additionally, acetic acid is added in response to increases in pH in the same manner as described in Example 1. The *C. cohnii* 30772 is allowed to grow in the 1 L fermenter for 72 hours after which the content of the 1 L fermenter forms the inoculum.

After this period, 700 ml of inoculum is taken from the 1 L fermenter and cultured

with the growth medium of Example 1 in the 5 L fermenter 10, exactly as described above in Example 1. During the culture in the 5 L fermenter, samples are taken at time intervals for analysis of dry cell weight and lipid content. The results of these analyses are shown in Figures 5 and 6, respectively. For comparison, Figures 5 and 6 also show comparable analyses on samples taken during the culture of *C. cohnii* 30772 during Example 1. As will be seen from Figures 5 and 6, both dry cell weight and lipid content increase more rapidly during culture in the 5 L fermenter 10 when the *C. cohnii* 30772 has been pre-grown with acetic acid/acetate (in Example 2), as opposed to being pre-grown with glucose as a carbon source (in Example 1).

### Example 3

In Example 3, *C. cohnii* 30772 is grown with acetic acid/acetate in the same manner as in Example 1, and with different concentrations of sea salts.

A first inoculum of *C. cohnii* 30772 is prepared as described above in Example 2. The first inoculum is then cultured in the 5 L fermenter 10, exactly as described in Example 1.

A second inoculum is prepared in the same way as the first inoculum. The second inoculum is then cultured in the 5 L fermenter 10, as described in Example 1, but with an initial concentration of sea salts in the growth medium of 16 g/L (rather than 25 g/L in Example 1).

During the culture of the first and second inocula, samples are taken at time intervals for the analysis of dry cell weight and lipid content. The results of these analyses are shown in Figures 7, 8 and 9. As will be seen from these Figures, culture of the *C. cohnii* 30772 with 16 g/L sea salts results in poorer lipid production compared to culture with 25 g/L sea salts.

#### Example 4

In Example 4, *C. cohnii* 30772 was cultured in seven separate batch cultures. Each culture was performed as described above in Example 1 with the exception that in six of the cultures, the initial sodium acetate concentrations in the growth media were, respectively, 4, 6, 8, 10, 12 and 16 g/L. In the seventh culture the initial sodium acetate concentration in the growth medium was 1 g/L (i.e. this culture was performed identically to the culture of *C. cohnii* 30772 in Example 1).

The results are shown in Figures 10 to 12. Figure 10 shows the dry cell levels (in g/L) at various time intervals over the 140 hour cultures. Figure 11 shows the lipid production (in g/L) at the same time intervals and Figure 12 shows the DHA production (in g/L) at the same time intervals. The highest levels of dry cells, lipid and DHA were obtained in the culture that used 8 g/L (initial concentration) sodium acetate in the growth medium. The lowest levels of dry cells, lipid and DHA were seen in the culture that used 16 g/L (initial concentration) in the growth medium. The levels of dry cells, lipid and DHA were broadly similar in the cultures that used 1, 4,

6 and 12 g/L sodium acetate.

The results may indicate that initial concentrations of sodium acetate of 16 g/L or above are inhibitory for growth of *C. cohnii* 30772.

#### Example 5

*C. cohnii* was cultured generally as described in Example 1, but using a growth medium containing 10 g/L yeast extract (compared to 7.5 g/L in Example 1) and 8.0 g/L sodium acetate (compared to 1 g/L in Example 1). The growth medium also contained 25 g/L sea salt as per the growth medium of Example 1.

As shown in Figure 13, it was found that increases in dry cell weight, total lipid and DHA levels continued up until 209 hours of culture (as opposed to 140 hours in Example 1), with higher overall levels of dry cell weight, total lipid and DHA being achieved after 209 hours than those achieved after 140 hours in Example 1. The dry cell weight, total lipid and total DHA content increased linearly between 72 and 209 hours of cultivation. A stationary phase was not necessary in order to obtain satisfactory production of DHA. The maximum overall volumetric DHA productivity was achieved after 209 hours of cultivation. The dry cell weight, total lipid and total DHA were then, 51.1, 27.6 and 8.0 g/L, respectively.

It is believed that the increased productive culture time and the increased levels of dry

cell weight, lipid and DHA may be caused by the additional yeast extract.

Figure 13 also shows that the percentage content of lipid in the cells increased over time, to a maximum of 54.0% at 209 hours. The DHA content of the extracted lipid varied between 30% and 40%, and was stable at about 30% between 67 and 239 hours.

Values of dry cell weight, total lipid content and DHA content are given at various time intervals in Table 3 which also gives various other values. It is notable that the overall volumetric production of DHA was 38.5 mg/l/h.

**Table 3**

time h	H Ac g/l	DCW g/l	lipid %	DHA %	tlipid g/l	tDHA g/l	QDHA mg/l/h
0	0.0	0.7	8.2	33.9	0.1	0.0	0.0
19	3.3	1.8	5.7	37.3	0.1	0.0	2.1
24	6.3	2.7	5.8	36.4	0.2	0.1	2.4
43	30.4	7.0	9.1	36.4	0.6	0.2	5.5
47	39.7	9.0	10.3	35.5	0.9	0.3	7.0
67	76.9	12.0	20.0	31.0	2.4	0.7	11.2
72	86.5	14.0	19.5	31.5	2.7	0.9	12.0
91	123.6	19.3	28.3	29.3	5.4	1.6	17.5
119	178.4	26.7	40.4	29.6	10.8	3.2	26.9
141	220.5	32.6	45.8	28.9	14.9	4.3	30.7
163	262.3	37.6	49.4	29.6	18.6	5.5	33.7
186	297.0	42.3	52.3	29.6	22.1	6.5	35.2

209	327.1	51.1	54.0	29.1	27.6	8.0	38.5
239	361.2	51.1	54.2	30.0	27.7	8.3	34.8

Abbreviations	Explanation
H Ac	Total amount of acetic acid added (in g)
DCW	Dry cell weight (in g/l)
lipid	Lipid content of the dry cells (in %)
DHA	DHA content of the lipid (in %)
tlipid	Total lipid concentration (in g/l)
tDHA	Total DHA concentration (in g/l)
QDHA	Overall volumetric productivity of DHA (in mg/l/h)

### Example 6

In a system where both acetic acid and glucose were fed it was demonstrated that acetic acid was used by *C. cohnii* and not glucose as the glucose added to the culture was still present in the medium. Apparently, *C. cohnii* prefers acetic acid above glucose and consumes only acetic acid when both acetic acid and glucose are offered.

The medium (800 ml) consisted of 10 g yeast extract, 25 g sea salt, 4.5 g glucose and 6.3 g sodium acetate. The inoculum (100 ml) was grown on 25 g/l sea salt, 6.2 g/l sodium acetate, 25 g/l glucose and 5.5 g/l yeast extract.

The feed was added via the pH control system described in Example 1 in order to maintain the pH at 6.5. The feed contained 273 g/l glucose and 280 g/l acetic acid.

Microorganisms cultured by the methods described above can be used as a food or food supplement. Before such use the microorganisms may be separated from the

culture medium and, if desired, subjected to processes such as drying, freeze-drying, etc. The cultured microorganisms may be particularly suitable as a food or food supplement for fish.

10030700-0140E

CLAIMS

1. A method of culturing a microorganism for the synthesis of docosaehaenoic acid by the microorganism, comprising culturing a microorganism selected from the group of *Crypthecodinium cohnii* and genetically modified variants thereof with a compound selected from the group of carboxylic acids and carboxylate ions, the microorganism using the compound as a carbon source and synthesising docosaehaenoic acid.
2. A method according to claim 1, wherein said compound is acetic acid or acetate.
3. A method according to claim 1 or claim 2, wherein the compound is the main carbon source for the microorganism during the culture of the microorganism.
4. A method according to any preceding claim, wherein the microorganism is cultured in a medium, said use of the compound as a carbon source by the microorganism causing an increase in pH of the medium, and wherein the method further includes, where the compound is a carboxylic acid, addition to the medium of said carboxylic acid, or where the compound is a carboxylate ion, addition to the medium of a carboxylic acid that ionises to form said carboxylate ion, in response to the increase in pH so as to decrease the pH of the medium.



5. A method according to claim 4, wherein said addition maintains the pH substantially at a predetermined value.
6. A method according to claim 5, wherein the predetermined value is pH 6.5.
7. A method according to any one of claims 4 to 6, wherein the pH of the medium is monitored by means that produces a signal that is used to control said addition to the medium.
8. A method according to claim 7, wherein the signal is used to control addition of one or more of a nitrogen source, a phosphorus source, an amino acid, a vitamin, a salt or another growth factor during the culture of the microorganism.
9. A method according to any one of claims 4 to 7, wherein said carboxylic acid or said carboxylic acid that ionises is added to the medium in a mixture comprising a further compound.
10. A method according to claim 9, wherein the further compound is an organic acid.
11. A method according to claim 9, wherein the further compound is a lipid.

12. A method according to any one of claims 9 to 11, wherein the mixture is a waste product from an industrial process.

13. A method according to claim 9, wherein the further compound is a nitrogen source, a phosphorus source, an amino acid, a vitamin, a growth factor, a salt or a lipid.

14. A method according to any one of claims 1 to 13, wherein prior to said culture with said compound, the microorganism is grown with said compound.

15. A method according to any one of claims 1 to 14, wherein the microorganism is cultured with an organic nitrogen source, preferably with yeast extract.

16. A method according to claim 15, wherein the nitrogen source is yeast extract and the initial concentration of the yeast extract is greater than 7.5 g/l.

17. A method according to claim 16, wherein the initial concentration of yeast extract is 10 g/l.

18. A method according to any one of claims 1 to 17, wherein the microorganism is cultured with salts or osmoticants, preferably with sea salts.

27

19. A method according to any one of claims 1 to 18, wherein said culture is performed as a batch process or a fed-batch process.

20. A method according to claim 19, wherein the culture is performed for between about 4 to about 10 days, preferably between about 6 to about 9 days.

21. A method according to any one of claims 1 to 18, wherein said culture is performed as a continuous process or semi-continuous process.

22. A method according to any one of claims 1 to 21, wherein the method further comprises extracting oil including docosahexaenoic acid from the microorganism and, preferably, purifying the oil to increase the docosahexaenoic acid content of the oil.

23. A method according to any one of claims 1 to 22, wherein the method further comprises the purification or partial purification of docosahexaenoic acid from the microorganism.

24. A method according to any one of claims 1 to 23, wherein the culture does not include a stationary phase.

25. An oil comprising docosahexaenoic acid prepared from a microorganism cultured in accordance with any one of claims 1 to 24.

26. An at least partially purified preparation of docosahexaenoic acid prepared from a microorganism cultured in accordance with any one of claims 1 to 24.
27. A method according to any one of claims 1 to 24, wherein the initial concentration of the compound is about 8 g/l.
28. A method of culturing a microorganism for the synthesis of docosahexaenoic acid by the microorganism, comprising culturing the microorganism with an organic species comprising an acidic group or an ionised form of an acidic group, the microorganism synthesising docosahexaenoic acid containing carbon from the species.
29. A method of culturing a microorganism for the synthesis of a polyunsaturated fatty acid by the microorganism, comprising culturing *C. cohnii* with an organic species comprising an acidic group or an ionised form of an acidic group, the *C. cohnii* using the species as a carbon source and synthesising a polyunsaturated fatty acid.
30. An oil comprising said polyunsaturated fatty acid of claim 29, prepared from a microorganism cultured in accordance with claim 29.
31. An at least partially purified preparation of said polyunsaturated fatty acid of claim 29, prepared from a microorganism cultured in accordance with claim 29.

29

32. A microorganism cultured in accordance with any one of claims 1 to 24, 28 or 29.

33. A method comprising using a microorganism according to claim 32 as a food or a food supplement.

[illegible]

Fig.1.

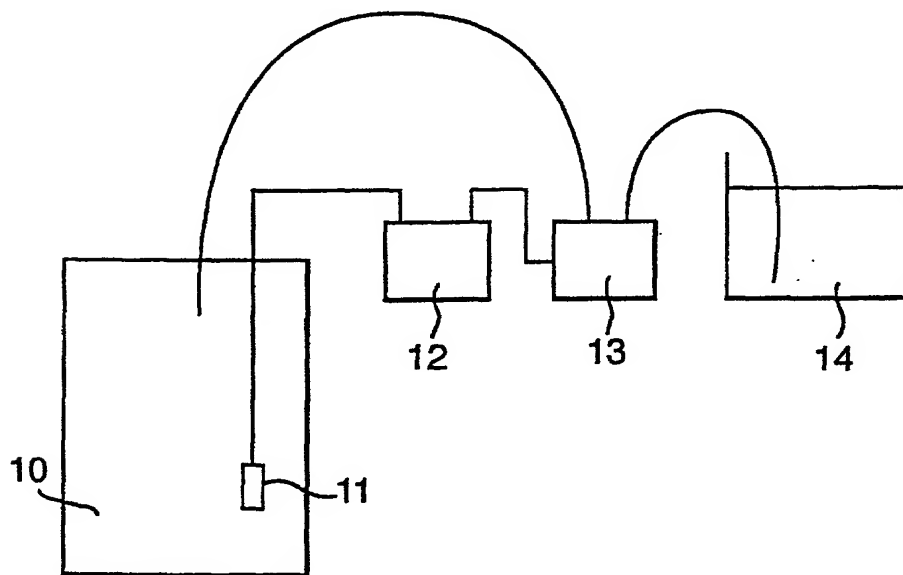


Fig.10.

Cultivation of *C. cohnii* ATCC 30772 on various sodium acetate concentrations

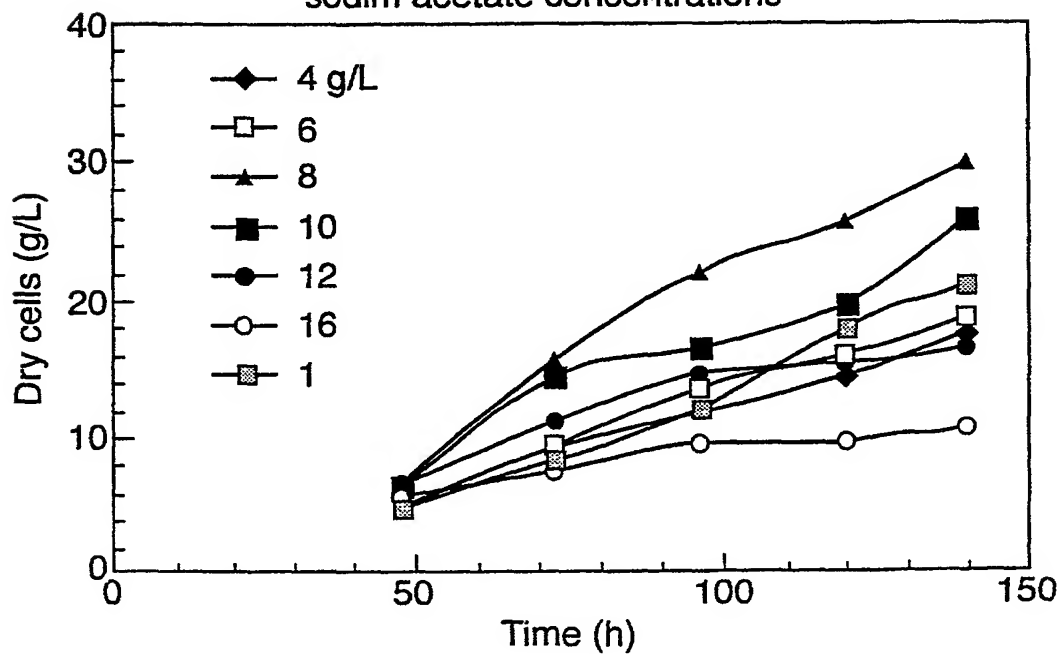


Fig.2.

*C. cohnii* ATCC 30772

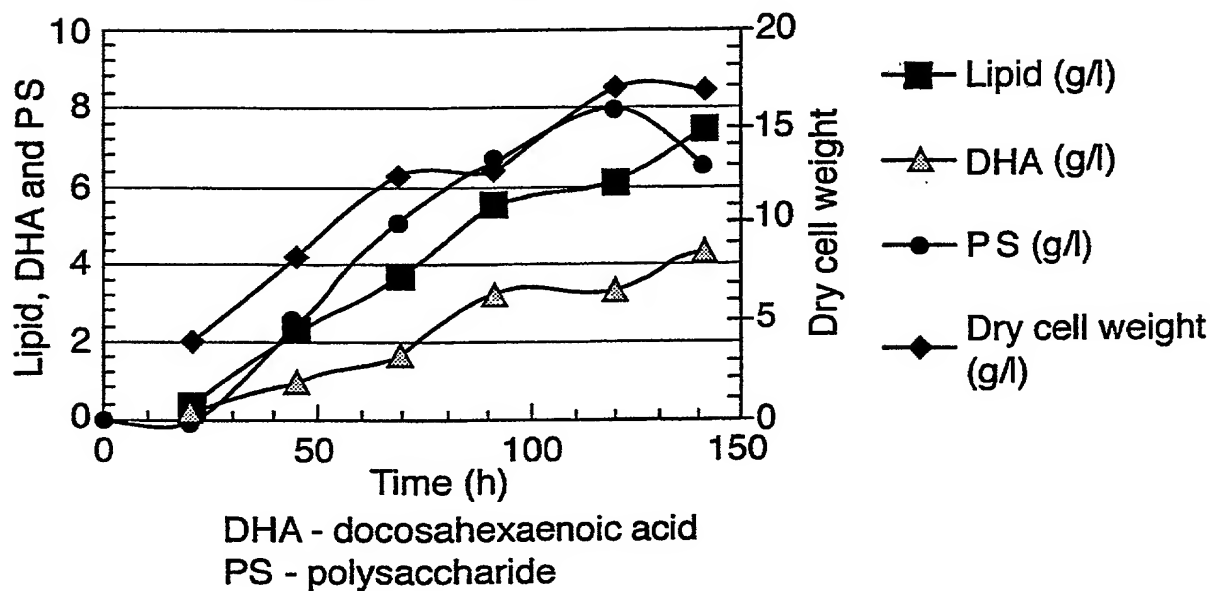
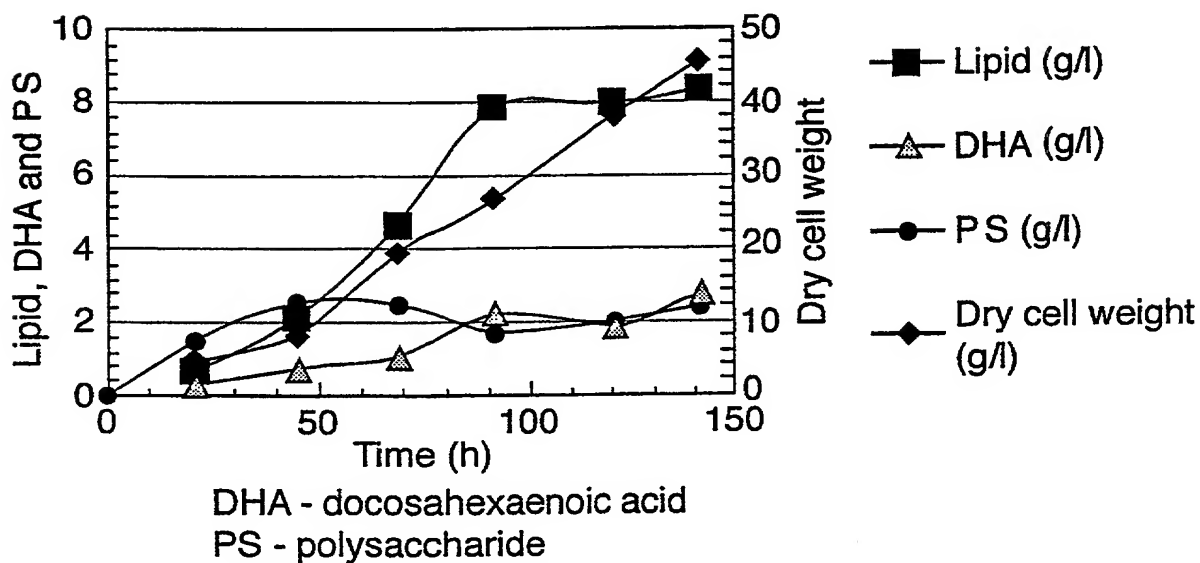


Fig.3.

*C. cohnii* ATCC 30541



3/7

Fig.4.

*C. cohnii* ATCC 50298

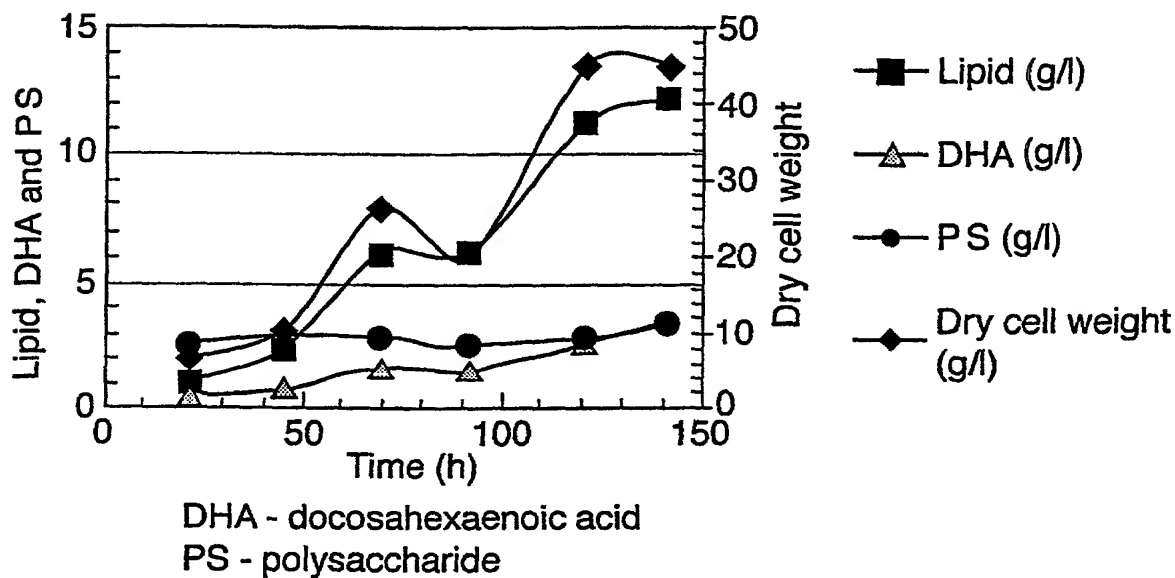


Fig.5.

Comparison of the dry cell weight of *C. cohnii* ATCC 30772 with glucose inoculum and acetate inoculum

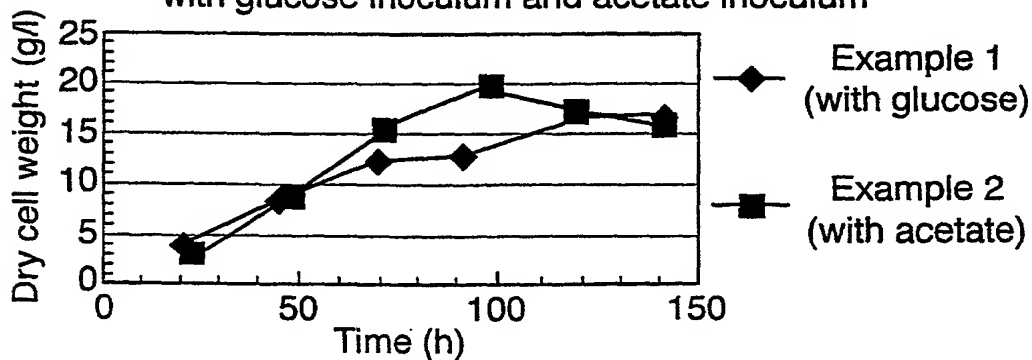




Fig.6.

Comparison of the lipid production of *C. cohnii* ATCC 30772 with glucose inoculum and acetate inoculum

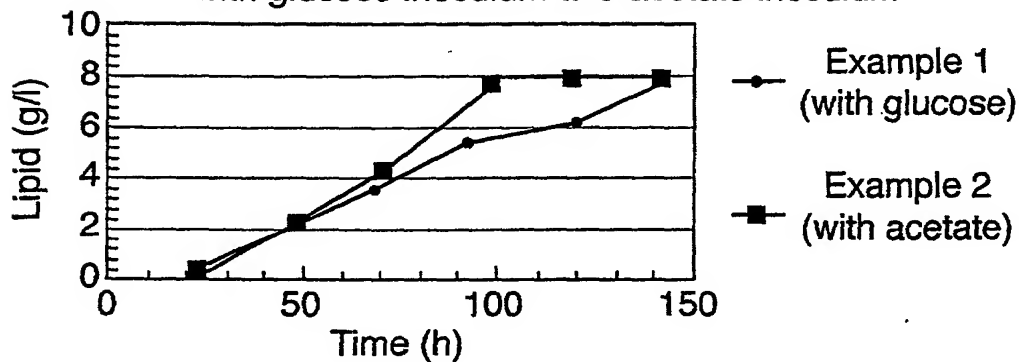


Fig.7.

Comparison of the dry cell weight *C. cohnii* ATCC 30772 on 25 g sea salts/L medium with 16 g sea salts/L medium

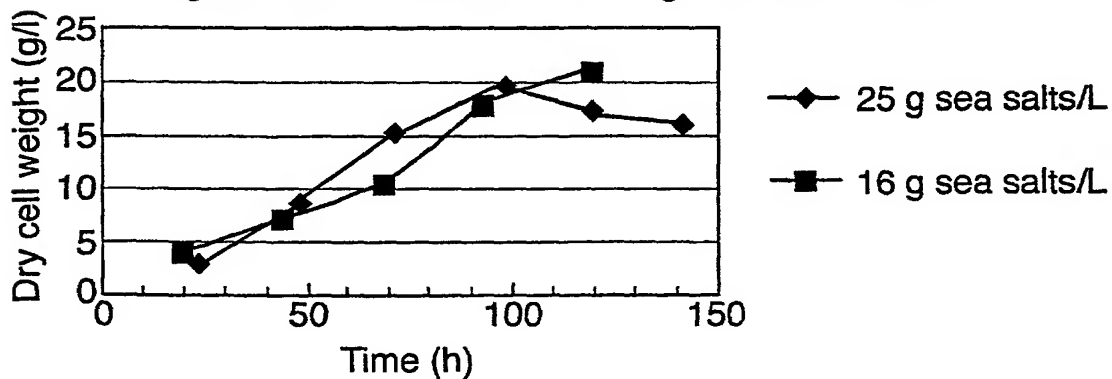


Fig.8.

Comparison of the lipid production of *C. cohnii* ATC 30772 on 25 g sea salts/L medium with 16 g sea salts/ L medium

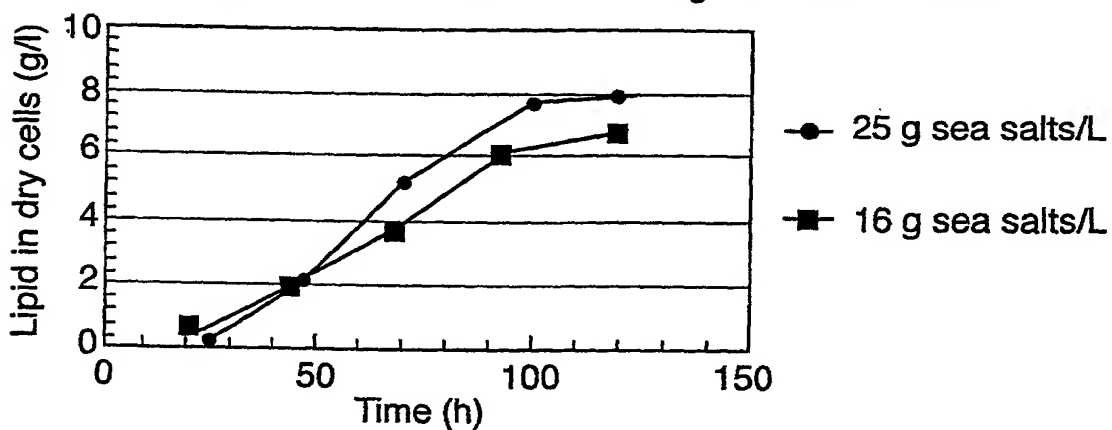


Fig.9.

Comparison of the lipid production of *C. cohnii* ATC 30772 on 25 g sea salts/L medium with 16 g sea salts/ L medium

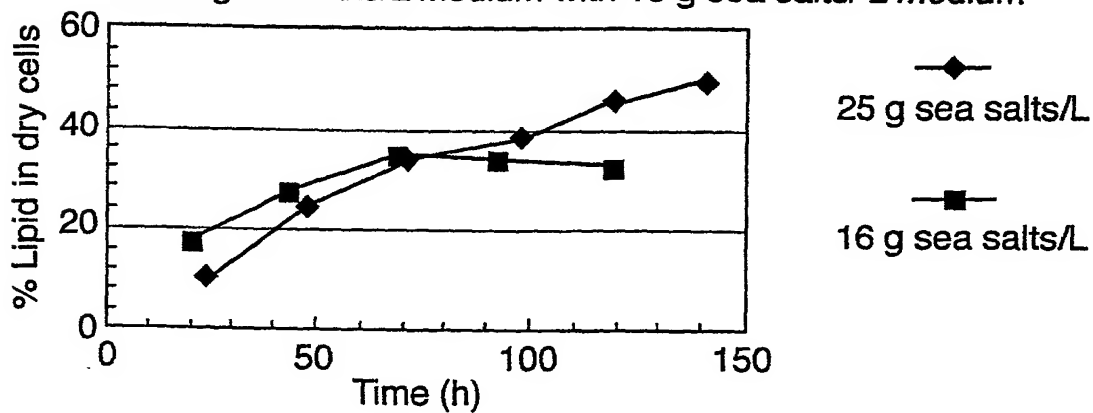


Fig.11.

Lipid production of *C. cohnii* ATCC 30772 on various  
sodium acetate concentrations

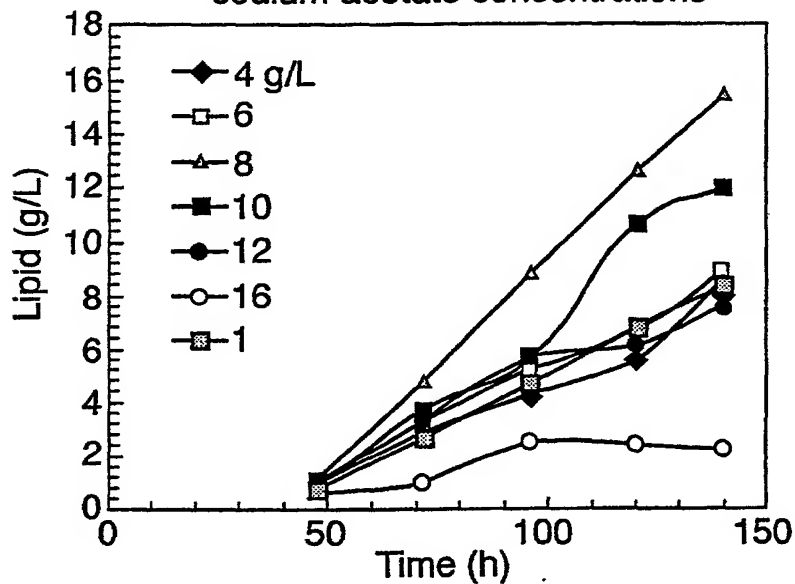


Fig.12.

DHA production of *C. cohnii* ATCC 30772 on various  
sodium acetate concentrations

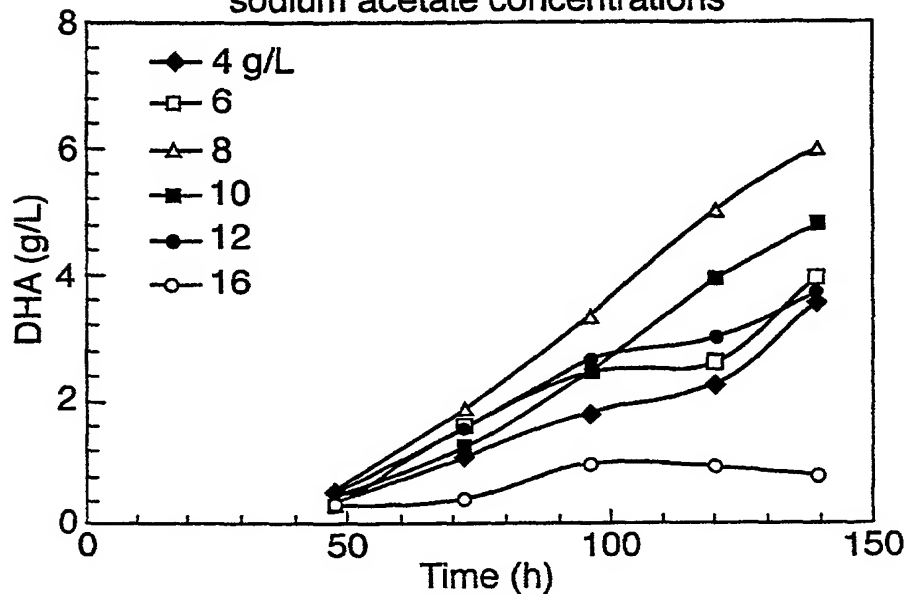
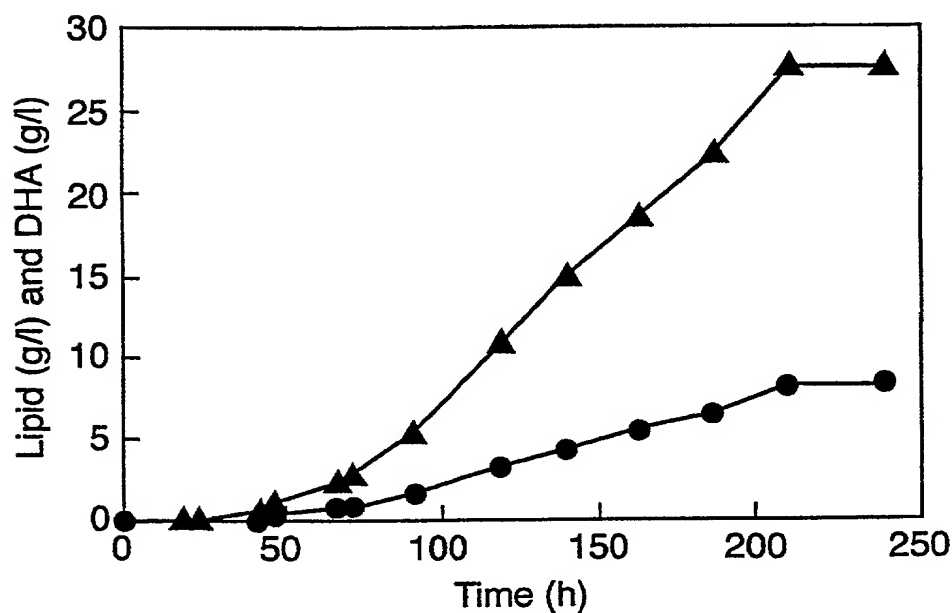
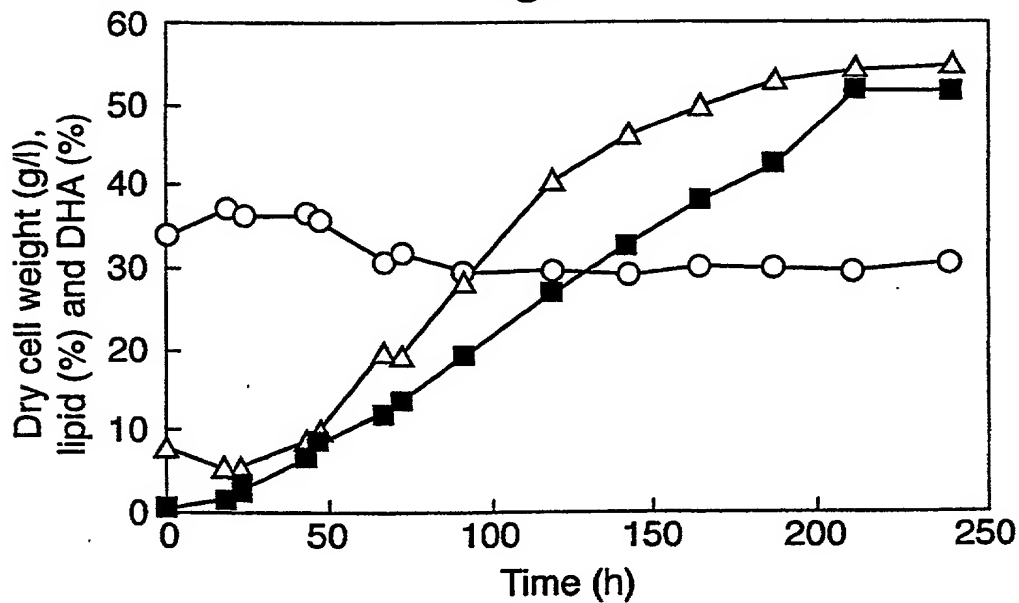


Fig.13.



*C. cohnii* cultivated in a 1L fermenter on 8.0 g/l NaAc, 10 g/l yeast extract, 25 g/l sea salt and as feed 50% (w/v) acetic acid added via the pH control system. The dry cell weight in g/l (■), lipid percentage of the cells (lipid in %) (Δ), DHA percentage of the lipid (DHA in %) (O), total lipid (Lipid in g/l) (▲) and DHA in g/l (●) are indicated.

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that.

This declaration is of the following type:

- ☐ original ☐ design ☐ supplemental  
☒ national stage of PCT  
☐ divisional ☐ continuation ☐ continuation-in-part

My residence, post office address, and citizenship are as stated below next to my name. I believe I am the original, first, and sole inventor (*if only one name is listed below*) or an original, first, and joint inventor (*if plural names are listed below*) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

"CULTURE OF MICROORGANISMS FOR THE SYNTHESIS OF A  
POLYUNSATURATED FATTY ACID"

the specification of which:

- ☐ is attached hereto.  
☐ was filed on \_\_\_\_\_ as Application No. \_\_\_\_\_ and was amended on \_\_\_\_\_ (*if applicable*).  
☐ was filed by Express Mail No. \_\_\_\_\_ as Application No. not known yet, and was amended on \_\_\_\_\_ (*if applicable*).  
☒ was filed on 13 July 2000 as PCT International Application No. PCT/GB00/02695 and was amended pursuant to PCT Article 19 on \_\_\_\_\_ (*if any*).

I state that I have reviewed and understand the contents of the specification identified above, including the claim(s), as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to the patentability of the application identified above in accordance with 37 CFR 1.56.

I claim foreign priority benefits under 35 USC 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate or 365(a) of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent, utility model, design registration, or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter and having a filing date before that of the application(s) from which the benefit of priority is claimed.

PRIOR FOREIGN PATENT, UTILITY MODEL, AND DESIGN REGISTRATION APPLICATIONS						
COUNTRY	PRIOR FOREIGN APPLICATION NO.	DATE OF FILING (day,month,year)	PRIORITY CLAIMED			
UNITED KINGDOM	9916537.5	14 July 1999	X	YES		NO
				YES		NO
				YES		NO

In re Appln of  
Attorney Docket No

I claim the benefit pursuant to 35 USC 119(e) of the following United States provisional patent application(s)

PRIOR U.S. PROVISIONAL PATENT APPLICATIONS, BENEFIT CLAIMED UNDER 35 USC 119(e)	
APPLICATION NO	DATE OF FILING (day,month,year)

I claim the benefit pursuant to 35 USC 120 of any United States patent application(s) or PCT international application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this patent application is not disclosed in the prior patent application(s) in the manner provided by the first paragraph of 35 USC 112, I acknowledge the duty to disclose material information as defined in 37 CFR 1.56 effective between the filing date of the prior patent application(s) and the national or PCT international filing date of this patent application.

PRIOR U.S. PATENT APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S., BENEFIT CLAIMED UNDER 35 USC 120					
U.S. PATENT APPLICATIONS			Status (check one)		
U.S. APPLICATION NO.	U.S. FILING DATE		PATENTED	PENDING	ABANDONED
1.					
2.					
3.					
PCT APPLICATIONS DESIGNATING THE U.S.			Status (check one)		
PCT APPLICATION NO.	PCT FILING DATE (day,month,year)	U.S. APPLICATION NOS. ASSIGNED (if any)	PATENTED	PENDING	ABANDONED
4. GB00/02695	13 July 2000			X	
5.					
6.					

DETAILS OF FOREIGN APPLICATIONS FROM WHICH PRIORITY CLAIMED UNDER 35 USC 119 FOR ABOVE LISTED U.S./PCT APPLICATIONS				
ABOVE APPLICATION NO.	COUNTRY	APPLICATION NO.	DATE OF FILING (day,month,year)	DATE OF ISSUE (day,month,year)
1. GB00/02695	UNITED KINGDOM	9916537.5	14 July 1999	
2.				
3.				
4.				
5.				
6.				

In re Appln of  
Attorney Docket No

As a named inventor, I appoint the following registered practitioner(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith Customer Number 23548.



**23548**

PATENT TRADEMARK OFFICE

I further direct that correspondence concerning this application be directed to Customer Number 23548



**23548**

PATENT TRADEMARK OFFICE

I declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor: <sup>1-00</sup> COLIN RATLEDGE

Inventor's signature Colin Ratledge

Date 2 May 2001

Country of Citizenship: UNITED  
KINGDOM

GBX

Residence Beverley, East Yorkshire, United Kingdom

Post Office Address: 49 Church Drive, Leven, Beverley, East Yorkshire,  
HU17 5LH, United Kingdom

Full name of second joint inventor, if any: <sup>2-00</sup> ALISTAIR JAMES ANDERSON

Inventor's signature A J Anderson Alistair James Anderson

Date 2 May 2001

Country of Citizenship: UNITED  
KINGDOM

GBX

Residence. Hull, United Kingdom

Post Office Address: 40 Oxenhope Road, Hull, HU6 7BZ, United Kingdom

In re Appln. of  
Attorney Docket No.

3-00  
Full name of third joint inventor, if any: KANAGASOORIYAM KANAGACHANDRAN

Inventor's signature K. Kanagachandran Kanagasooriyam Kanagachandran

Date 14 May 2001

Country of Citizenship SRI LANKA

Residence: London, United Kingdom GB

LKX

Post Office Address: 49 Trevelyan Road, London, SW17 9LR, United Kingdom

4-00  
Full name of fourth joint inventor, if any: DAVID JOHN GRANTHAM

Inventor's signature David John Grantham David John Grantham

Date 02/05/2001

Country of Citizenship: UNITED KINGDOM GBX

Residence: Hull, East Yorkshire, United Kingdom

Post Office Address: 2 Malvern Crescent, Priory Road, Hull, East Yorkshire, HU5 5TR, United Kingdom

5-00  
Full name of fifth joint inventor, if any: JANET CHRISTINE STEPHENSON

Inventor's signature Janet Christie Stephenson Janet Christie Stephenson

Date 2 May 2001

Country of Citizenship: UNITED KINGDOM GBX

Residence: Hull, East Yorkshire, United Kingdom

Post Office Address: 6 St. Anthony's Drive, Hedon, Hull, East Yorkshire, HU12 8NT, United Kingdom

6-00  
Full name of sixth joint inventor, if any: MARTIN E. de SWAAF

Inventor's signature Martin E. de Swaaf ME de Swaaf

Date 29 May 2001

Country of Citizenship: THE NETHERLANDS NLX

Residence: Utrecht, The Netherlands

Post Office Address: Dillenburgstraat 27, 3583 VD Utrecht, The Netherlands



In re Appln. of  
Attorney Docket No.

Full name of <sup>7-00</sup> joint inventor, if any LOLKE SIJTSMA

Inventor's signature Lolke Sijtsma Sijtsma

Date 29<sup>th</sup> May 2001

Country of Citizenship.

THE  
NETHERLANDS

Residence: Renkum, The Netherlands

NLX

Post Office Address: Brinkweg 18, 6871 VK Renkum, The Netherlands

10030700 "04402  
20470 0070E00T